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14. ABSTRACT
The purpose of this concept award grant is to uncover potential drug targets for treatment of Neurofibromatosis type 2 (NF2). We planned a synthetic lethal screen, using RNAi technology to uncover protein kinases and phosphates that are specifically required for the survival of NF2-null cells. We also obtained and reformatted a murine siRNA library against all known protein kinases and phosphatases. We also obtained NF2flox/flox mouse embryo fibroblasts and used Cre recombinase to convert these to a NF2-/- genotype. We then tested a large number of transfection method worked well on the mouse embryo fibroblasts. We then obtained a library of bioactive chemicals and carried out a screen for synthetic lethality. This screen revealed that an inhibitor of the hedgehog pathway (cyclopamine), an inhibitor of Src-family protein kinases (damnacanthal, as well as PP1, PP2, and tyrphostin), and an agonist of the constitutive androstane receptor (CITGO), all were selectively toxic to NF2-/- cells. These findings provided the basis for obtaining a grant from the Children's Tumor Foundation.

15. SUBJECT TERMS
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INTRODUCTION

The purpose of this concept award grant is to uncover potential drug targets for treatment of NF2. Specifically, we had planned a synthetic lethal screen, using RNAi technology to uncover protein kinases and phosphatases that are specifically required for the survival of NF2-null cells.

BODY

As modified in last year's progress report, we had set ourselves four specific tasks. These were:

Task 1. Obtain and reformat mouse siRNA kinase/phosphatase library:

a. Prepare two replica plates of library, pooling the two siRNAs against each kinase into single well.

Task 2. Test transfection methods to achieve adequate (>70%) knock-down of test genes:

a. Transfect immortalized GFP-expressing MEFs with an siRNA directed against GFP. Measure loss of GFP expression and viability of cells.
b. Test efficacy of selected retroviral shRNAs against kinases thought to be germane to NF2 signaling, e.g., Pak1, Pak2, and MLK-3.
c. Standardize assay for 96-well format and obtain a Z' score.

Task 3. Convert $NF2^{flox/flox}$ MEFs to $NF2^{-/-}$ genotype:

a. Infect $NF2^{flox/flox}$ MEFs with a retrovirus expressing Cre recombinase and YFP, sort for YFP positive cells

Task 4. Screen siRNA kinase/phosphatase library for effects on growth and morphology of *WT* and *NF2-null* MEFs:

a. Measure density of growth in the presence or absence of transfected siRNAs.
d. Retest positives from Task 4a to confirm phenotypic effects.
e. Test for physical interaction of proteins targeted by shRNA and Merlin.

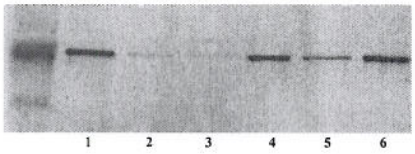

Fig. 1. Efficacy of siRNA. $NF2^{-/-}$ cells were transfected with Pak2 siRNA to final concentration of 10 nmol/l. An immunoblot for Pak2 was performed 48 hr post transfection. 1) OligoFectAmine; 2) LipoFectAmine2000; 3) HiPerFect (serum-free media); 4) HiPerFect (full media); 5) RNAiFect; 6) nontransfected cells.

Progress

Tasks 1, 2, and 4. There was a major change in design in this study. We found that the immortalized MEFs, whether *WT* or $NF2^{-/-}$, were extremely resistant to transfection by RNAi. We tested a dozen different methods, including lipofectamine, lipofectamine 2000, oligofectamine, Dreamfectin, HiPerfect, and RNAifect, and found that many of these did not efficiently transfect the MEFs. In the few cases where efficient transfection was achieved (*e.g.*, Lipofectamine 2000 and HiPerfect in low serum conditions) (**Fig. 1**), it came invariably at the cost of excessive toxicity (not shown). Since our assay involves measurements of cell viability as a read-out, none of these lipid based transfection methods was acceptable. Very recently, the Fox Chase Cancer Center acquired a 96-well electroporation unit from Amaxa, which is reputed to work well on many recalcitrant, hard-to-transfect cells, but we have not yet tested if this method will serve our needs.

For these reasons, we altered our plan for a synthetic lethal screen from the kinase/phosphatase RNAi library to a small molecule library. That is, we tested a library of bioactive compounds to identify those that are more toxic to $NF2^{-/-}$ cells than to wild type cells. By using small molecules, we could avoid the issue of transfection efficiency. The challenge for small molecule screens is, rather, to verify the molecular target(s) responsible for any biological activities that are found.

The two cell types (*WT* and $NF2^{-/-}$ MEFs) were grown to confluence and then treated in duplicate with each of 560 compounds (10 uM) from the Biomol known Bioactives (480 diverse biologically active compounds with defined biological activity) collection (http://www.biomol.com/Online_Catalog/Online_Catalog/Products/Product_Detail/38/?categoryId=405&productId=2483&mid=75). After three days, viability was assessed using a modified MTT assay. Most compounds had equivalent effects on viability irrespective of *Nf2* status. However, a number of compounds were selectively toxic (**Fig. 2**) in one or the other genetic background. Naturally, we are more interested in those that are toxic to NF2 cells rather than those that are toxic to normal cells. Of particular interest are cyclopamine, an inhibitor of the hedgehog pathway [1], damnacanthal, an inhibitor of Src-family protein kinases (N.B, other Src inhibitors were also identified: PP1, PP2, and Tyrphostin), and CITCO, an agonist of the constitutive androstane receptor [2]. This latter compound was extremely selective in killing $Nf2^{-/-}$ cells. We are currently retesting these compounds plus inactive controls.

Task 3. As reported last year, we obtained $NF2^{flox/flox}$ MEFs from Andi McClatchey (Harvard Medical School). These were left as is or converted to *NF2*□ by infection with Adeno GFP (control) or Adeno Cre-GFP, respectively. Cells were sorted twice for GFP, then tested for genotype and phenotype as described in last year's report. Our results show that removal of

Drugs toxic to *NF2*$^{-/-}$ cells (synthetic lethals)			Drugs toxic to *NF2*$^{+/+}$cells			
NS-1619	33%	(stimulation of nf2+/+)	Diltiazem	66%	Calyculin A	46%
Boc-GVV-CHO	33%		Nimodipine	50%	Camptothecin	43%
Pinacidil	33%	(stimulation of nf2+/+)	Pimozide	46%	Cantharidin	41%
D609	37%		Tosyl-Phe-CMK (TPCK)	81%	Cholerythrine	43%
Damnacanthal	71%		Thiorphan	60%	CAPE	54%
Deoxymannojirimycin (1)	34%		NapSul-Ile-Trp-CHO	41%	Geldanamycin	35%
H-89	33%		SDZ-201106	37%	Ionomycin	40%
HBDDE	34%		2-Methoxyantimycin A3	40%	NSC-95397	40%
L-NAME	48%		Betulinic acid	35%	Lavendustin A	34%
Monensin	30%	(stimulation of nf2+/+)	10-Hydroxycamptothecin	34%	Tyrphostin -8	38%
Phenanthridinone -Phenanthridinone]	47%	(stimulation of nf2+/+)	β-Lapachone	36%	Manoalide	55%
CITCO	92%		Parthenolide	33%	Manumycin A	38%
Cyclopamine	71%		MG-132	37%	Gliotoxin	39%
PP1	60%		Helenalin	52%	GW-5074	48%
RWJ-60475-(AM)3	43%		Bezafibrate	36%	Shikonin	35%
PP2	39%		Ascomycin (FK-520)	41%		
Thalidomide	53%		Zaprinast	50%		
Trichostatin -A	38%		MY-5445	53%		
Trifluoperazine	41%		Ikarugamyin	36%		
Tyrphostin 1	58%		MBCQ	35%		
Valinomycin	35%		Bafilomycin A1	35%		
Z-VAD-FMK	66%		BAPTA-AM	39%		
AA-861	38%		CA-074-Me	38%		

Fig. 2. Screening of 480-member Biomol "Bioactives" library against *NF2*$^{-/-}$ and *NF2*$^{+/+}$ cells. Cells were grown to confluence, then treated with 10 uM compound for three days, followed by a measure of viability. Compounds that showed > 30% selective effects in either cell type are shown. Numbers represent % loss of viability relative to control.

NF2 profoundly alters cell proliferation, as expected for a tumor suppressor. This task was successfully completed as planned.

Task 4. We set ourselves a new task, to reflect follow-up on the small molecule-based synthetic lethal screen. To this end, we are currently performing dose-response curves using the two cell lines and our three best hits: CITCO, cyclopamine, and damnacanthal. We have also obtained from Johns Hopkins University, a library of FDA approved compounds [3]. We intend to repeat our screen with this chemical library. The idea behind this screen is that any hits will be, by definition, FDA approved drugs. This would eliminate the need for drug development.

KEY RESEARCH ACCOMPLISHMENTS:

- Found several compounds that are selectively toxic to cells lacking NF2.

REPORTABLE OUTCOMES

We have found a number of compounds that show selective toxicity in *NF2*$^{-/-}$ fibroblasts. These include an inhibitor of the hedgehog pathway (cyclopamine), an inhibitor of Src-family protein kinases (damnacanthal, as well as PP1, PP2, and tyrphostin), and an agonist of the constitutive androstane receptor.

These findings provided the basis for obtaining a grant from the Children's Tumor Foundation (Grant ID 2007B-05-003; *Synthetic lethal screen of FDA-approved drugs in*

NF2). We will continue to explore the activity of these and related compounds in NF2 model cells and organisms.

CONCLUSION

While we had to make considerable changes in the design of this study, our main goal was to find weaknesses in *NF2*-null cells that could be exploited therapeutically. I believe we have accomplished this goal. We have demonstrated the feasibility of the synthetic lethal screen, and shown that $NF2^{-/-}$ cells are susceptible to killing by several compounds that are relatively less toxic to normal cells.

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None.

LIST OF KEY PERSONNEL

Zahara Jaffer – Postdoctoral Associate